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Inhibition of Angiogenesis by the Cancer Chemopreventive Agent Conjugated Linoleic Acid[1]

Patricia A. Masso-Welch,[2] Danilo Zangani,[3] Clement Ip, Mary M. Vaughan, Suzanne Shoemaker, Robert A. Ramirez, and Margot M. Ip

Departments of Pharmacology and Therapeutics [P. A. M-W., D. Z., M. M. V., S. S., R. A. R., M. M. I.] and Cancer Prevention [C. I.], Roswell Park Cancer Institute, Buffalo, New York 14263

ABSTRACT

Dietary conjugated linoleic acid (CLA) has been shown previously to inhibit rat mammary carcinogenesis. In addition to direct effects on mammary epithelial cells, including decreased proliferation and induction of apoptosis, CLA may exert its effects indirectly by inhibiting the differentiation of mammary stromal cells to an endothelial cell type. Specifically, CLA was found to decrease the ability of mammary stromal cells to form complex anastomosing microcapillary networks *in vitro* on Engelbreth-Holm-Swarm-derived reconstituted basement membrane. This suggested that CLA might inhibit angiogenesis *in vivo*. To test this possibility, CD2/F_1 mice were placed on synthetic diets containing 0, 1, or 2% CLA for 6 weeks, before angiogenic challenge by s.c. injection with an angiogenic gel substrate (Matrigel pellet assay). After 7 days, the pellets from animals fed the control diet were infiltrated by abundant branching networks of blood vessels with patent lumen-containing RBCs. In contrast, pellets from the CLA-fed animals contained fewer infiltrating cells, which formed limited branching cellular networks, the majority of which had collapsed lumen and no RBCs. Both levels of dietary CLA showed similar effects, with the number of RBC-containing vessels per 20× field decreased to a third of that seen in control. Dietary CLA decreased serum levels of vascular endothelial growth factor (VEGF) and whole mammary gland levels of VEGF and its receptor Flk-1. Both *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers were effective in inhibiting angiogenesis *in vitro* in a dose-dependent fashion. The ability of CLA to inhibit angiogenesis may contribute to its efficacy as a chemopreventive agent.

INTRODUCTION

CLA[4] is a series of geometric and positional isomers of octadecadienoic acid that are present in meat and dairy products (reviewed in Ref. 1). This fatty acid has been shown to be effective at inhibiting carcinogenesis in multiple systems and at several levels, including initiation (2–6), promotion (7–9), progression (10), and metastasis (11–13). One of the ways in which CLA may mediate its effects in decreasing mammary carcinogenesis is through its effects on the normal mammary epithelium. CLA decreases lateral branching of the ductal tree (14) and decreases the labeling index of the terminal ductal lobular unit (8). The terminal end bud, the precursor to the terminal ductal lobular unit and the carcinogen-sensitive target in the nonparous rodent mammary gland (15), also shows a decreased proliferative index in response to dietary CLA (14), resulting in decreased terminal end bud number (16, 17). *In vitro* studies with primary cultured rat mammary epithelial cells have confirmed the inhibitory effect of CLA on their proliferation and demonstrated that CLA additionally induces apoptosis of these cells (18).

Potential modulation of the mammary stroma by CLA is of interest for several reasons. CLA can inhibit proliferation of preadipocytes (19), affect lipid accumulation (19–27), and induce apoptosis of adipocytes (24, 25). The ability of CLA to affect proliferation, differentiation, and apoptosis of stromal cells is of interest because mammary stromal cells are capable of undergoing multiple differentiation pathways in a hormone- and substratum-dependent manner, resulting in a fibroblast, adipocyte, or endothelial phenotype (28). This previously undescribed connection between these cell phenotypes led us to hypothesize that CLA might be effective in modulating angiogenesis *in vivo* through its effects on the differentiation of mammary stromal vascular precursors. Direct effects of CLA on tumor stroma *in vivo* could contribute to the ability of CLA to reduce metastasis and induce central necrosis of human prostate cancer grown in SCID mice (11). The ability of mammary stromal cells or their secreted products to influence the growth of normal breast and breast cancer has been well-documented (reviewed in Refs. 29, 30).

The purpose of this study was to determine the effects of dietary CLA on angiogenesis *in vivo*. This was done by analyzing the recruitment of endothelial precursors to a s.c. injected angiogenic EHS-RBM pellet (31, 32). Systemic and local effects of dietary CLA on VEGF and its receptors were analyzed. The concentration- and isomer-dependent effect of CLA on capillary formation by stromal vascular precursors *in vitro* was also assessed.

MATERIALS AND METHODS

CLA from Nu-Chek Prep, Inc. (Elysian, MN), containing 85–88% *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA at an approximate 1:1 ratio, as well as trace amounts of other isomers, was used in the *in vivo* studies and in the *in vitro* studies, where noted. For *in vitro* studies, highly purified (>95%) CLA isomers from Natural ASA (Hovdebyda, Norway) were used. Heparan sulfate, 3,3′,5,5′-tetramethyl benzidine liquid ELISA substrate, and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co. (St. Louis, MO). bFGF was obtained from Biodesign (Kennebunk, ME). The VEGF ELISA kit was purchased from R&D Systems (Minneapolis, MN). Rabbit antisera against VEGF-A (SC-7269) and Flk-1 (SC-505) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-hsc70 antibody (SPA-820) was obtained from Stressgen (Victoria, British Columbia, Canada). Leupeptin was purchased from Boehringer-Mannheim (Indianapolis, IN), and soybean trypsin inhibitor was from Calbiochem (La Jolla, CA). ECL (detection substrate used for hsc70 Western blots) and ECL-plus (detection substrate used for Flk-1 and VEGF Western blots) were obtained from Amersham (Arlington Heights, IL). Donkey antirabbit and antimouse secondary antibodies conjugated to HRP were purchased from Jackson Immunoresearch Laboratories (Westgrove, PA). Trans-blot (pure nitrocellulose) Transfer Membrane was purchased from Bio-Rad (Hercules, CA). Reconstituted basement membrane was extracted from the EHS mouse sarcoma as described previously (33).

Animal Care. CD2/F1 mice were purchased from the National Cancer Institute Frederick Cancer Research Facility, Biological Testing Branch (Frederick, MD), and were given water *ad libitum*. Animals were fed semisynthetic AIN-76A diets containing 5% (w/w) corn oil, without or with supplementation

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[2] To whom requests for reprints should be addressed, at Department of Pharmacology and Therapeutics, Grace Cancer Drug Center, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Phone: (716) 845-3126; Fax: (716) 845-5865; E-mail: patricia.masso-welch@roswellpark.org.

[3] Present address: Universita degli Studi di Sassari, Dipartimento di Scienze del Farmaco, Via Muroni 23/a, 07100 Sassari, Italy.

[4] The abbreviations used are: CLA, conjugated linoleic acid; LA, linoleic acid; EHS-RBM, Engelbreth-Holm-Swarm sarcoma-derived reconstituted basement membrane; bFGF, basic fibroblast growth factor; HRP, horseradish peroxidase; SCID, severe combined immunodeficient; VEGF, vascular endothelial growth factor; hsc70, heat shock cognate protein 70.

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with 1 or 2% CLA as described previously (34). CD2/F_1 mice, fed a chow diet, were also used to carry the EHS sarcoma (33). This tumor has been passaged in CD2/F_1 mice in our laboratory for the past 15 years. Animal rooms were air-conditioned and humidity controlled, with a light cycle of 12 h on and 12 h off. Animals were housed in accordance with the standards set by the NIH and the Roswell Park Cancer Institute Animal Care and Use Committee.

***In Vivo* EHS-RBM Angiogenesis Assay.** To determine the effects of dietary CLA on angiogenesis, the *in vivo* angiogenesis model of Pauly *et al.* (32) was used. Briefly, CD2/F_1 female mice were placed on diets with or without CLA at 8 or 12 weeks of age, with 10–12 mice/group/experiment. After 6 weeks of diet, mice were given bilateral injections of EHS-RBM containing 1.25 μg/ml bFGF and 60 μg/ml heparan sulfate using a 25-gauge needle, into the region of mammary gland four. Mice were sacrificed 7 days after injection. Blood was collected from the trunk for preparation of plasma for analysis of VEGF levels. Pellets and adjacent normal mammary gland tissue were removed and formalin fixed for paraffin embedding. This experiment was repeated four times.

Alternately, for analysis of whole mammary gland levels of VEGF and Flk-1, 10 mice/group were placed on diet at 8 weeks of age, and mice were sacrificed 7 weeks after diet initiation. Mammary glands were snap-frozen in liquid nitrogen to be used for preparation of whole gland lysates.

Analysis of Cellular Invasion. Microsope visual fields, visualized with a 20× objective, were analyzed for cellular invasion, and with a 40× objective for the presence of functional blood vessels, for a final magnification of ×200 or ×400, respectively. The ability of dietary CLA to affect cellular invasion into the EHS-RBM pellet was analyzed on H&E-stained paraffin sections by counting the number of nuclei/field, excluding all acellular regions that remained uninvaded. A total of 68 fields were assessed in 13 pellets from mice on control diets, 75 fields were assessed from 9 pellets from mice on 1% CLA diets, and 62 fields analyzed from 13 pellets, obtained from mice on 2% CLA diets. We analyzed all pellets retrieved from the animals; there was a decline in the number of visible pellets in CLA-fed animals.

Analysis of Functional Blood Vessel Formation. Functional angiogenesis was quantified by determining the number of cell-lined structures with patent lumen and RBCs. H&E-stained paraffin sections were visualized by epifluorescent illumination; RBCs are strongly fluorescent because of their intensely eosinophilic cytoplasm. This intensity is easily distinguished from the dimly fluorescent nucleated cells. The relative dimness of nucleated cells may be partly attributable to the presence of nuclear and/or cytoplasmic hematoxylin, which quenches fluorescence. A total of 162 fields were analyzed from 21 slides from control diet animals, 158 fields were analyzed from 21 slides from 1% CLA diet animals, and 174 fields were analyzed from 13 slides from 2% CLA diet animals.

Serum VEGF Analysis. Serum VEGF (VEGF-A) levels were assessed by ELISA using a commercial kit (Quantikine M mouse VEGF ELISA; R&D Systems), as per the manufacturer's instructions. VEGF levels were assessed in sera from mice that had been on diet for a total of 7 weeks and had received EHS-RBM angiogenic challenge 1 week before sacrifice. These data represent the results of four individual experiments, with 10 or more animals/diet/trial.

Analysis of VEGF and Flk-1 Expression in Mammary Gland Lysates. Whole mammary glands (gland four) were dissected after sacrifice from mice fed control- or CLA-supplemented diets, snap-frozen in liquid nitrogen, and stored at −80°C. Lysates were prepared by pulverizing the frozen glands in a liquid nitrogen-cooled metal pulverizer to a fine powder. The powder was weighed in a pretared microcentrifuge tube and resuspended in ice-cold Triton X-100 lysis buffer [50 mM Tris (pH 7.5 at room temperature, 250 mM NaCl, 5 mM EDTA, 0.1% (v/v) Triton X-100, supplemented with 0.1 mM phenylmethylsulfonyl fluoride, 20 ng/ml leupeptin, and 100 ng/ml soybean trypsin inhibitor] using 2 ml/g of tissue. The powder was then homogenized in an ice-cold microcentrifuge tube, using 10–15 strokes of a fitted glass homogenizer. Samples were incubated on ice for 30 min and then centrifuged for 10 min at 4°C at 8000 × *g*. The supernatants were then aliquoted and frozen at −20°C. An aliquot of the sample was used to determine protein concentration, using the Bio-Rad protein assay. Immediately before electrophoresis, samples were prepared for loading by adding 4× Laemmli SDS-sample buffer (35) at a 1:3 ratio to the thawed sample. The sample was then boiled, sonicated, and centrifuged for 10 min at 4°C, 8000 × *g* in a microcentrifuge, before loading supernatant. Lysates from seven mice/dietary group were analyzed.

Lysates were loaded using equal protein/well, and proteins were separated on a 7.5% reducing SDS-gel according to the method of Laemmli (35). Proteins were transferred to nitrocellulose using a Bio-Rad Transblot Apparatus (Hercules, CA). Blots were blocked with Blotto [TBS (10 mM Tris, pH 8.0, 150 mM NaCl), 0.5% Tween 20, 5% (w/v) nonfat dry milk]. All antibody incubations and subsequent rinses were performed in Blotto, with vigorous rocking. Rabbit anti-VEGF-A was used at a final concentration of 2 μg/ml, and rabbit anti-Flk-1 was used at a final concentration of 1 μg/ml. To control for variability in loading, the same blots used for VEGF were stripped and probed for Flk-1. The blots were then stripped again and probed with mouse antisera to hsc70 at a 1:20,000 final concentration. hsc70 was used as a control for cellular protein loading of Western blots, as a constitutively expressed cytoplasmic housekeeping protein. Because dietary CLA causes substantial changes in the cellular and extracellular composition of the mouse mammary gland,[5] total protein loading by Bio-Rad assay is not descriptive of the cellularity of the whole gland lysate. After binding and washing 5 × 5 min in Blotto, secondary HRP-conjugated donkey antirabbit or donkey antimouse antibodies were added at 1:5000 in Blotto. Blots were washed 5 × 5 min in Blotto, then 3 × 3 min with TBS before detection of HRP-conjugated secondary antibody using ECL (hsc70) or ECL-plus (VEGF and Flk-1) and exposure of Kodak X-Omat X-ray film. Specific antibody-reactive bands were quantified by densitometry using a Model 300A Scanning Laser Densitometer and ImageQuant software (Molecular Dynamics).

Effects of CLA on Tubule Formation *in Vitro*. An *in vitro* angiogenesis assay, the formation of microcapillary networks by rat mammary stromal vascular precursor cells on the angiogenic substrate, EHS-RBM, was used to compare the isomer- and concentration-dependent effects of mixed CLA isomers and *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA. Rat mammary stromal cells have the ability to act as true vascular precursors and were isolated from mammary glands as described (28).

CLA-containing medium was prepared as follows. Briefly, 18.1 μl of 5 N NaOH were added to 28.2 μl of CLA (0.9 g/ml; FW 280) in a small Erlenmeyer flask, followed by vortexing, to make the sodium salt. Warm FCS (10 ml) was added, sonicated three to four times with brief pulses (5 s each), and then gassed with nitrogen, and the flask was sealed with parafilm. The mixture was incubated in a sonicating water bath for 30 min, followed by 5 min incubation in a 50°C water bath. FBS (11.9 ml) was added to the flask, to bring the CLA to a final 33× concentration (4.125 mM). This 33× CLA stock solution was added to F12/DME stock and filtered through a 0.22 μM filter to make a sterile 125 μM stock, which was then used to prepare the other media concentrations, such that all CLA media had a final total concentration of 3% FBS.

Early-passage mammary stromal cells (before passage 4) were cultured in a 24-well plate at 2×10^4 cells/well, plated onto 0.4 ml of EHS-RBM in the presence of phenol red-free F12/DME with 3% FCS, supplemented with 0, 25, 50, 75, 100, or 125 μM CLA. After 7 days in culture, when the cells had formed multiple colonies of three-dimensional networks of tubules (28), capillary networks were photographed, and the extent of network formation was assessed by measuring four planar diameters, at 45° intervals, of the branching colonies. The five largest colonies in each of three wells/group were assessed, for a total of 15 colonies for each concentration of CLA per experiment. This experiment was replicated twice.

Statistics. Data were analyzed using SigmaStat 2.0 (Jandel Scientific). Analysis of serum VEGF and pellet invasion, cellularity, blood vessel formation, and *in vitro* capillary network formation were analyzed by performing ANOVA for parametric data (serum VEGF levels) and Kruskal-Wallis ANOVA on Ranks for nonparametric data (cell invasion, cellularity, and functional angiogenesis of EHS-RBM pellet). For Western blot analysis of whole mammary gland lysates, Tukey Multiple Comparisons was done for parametric data; Dunn's Multiple Comparisons and/or All Pairwise Multiple Comparison Procedures (Dunnett's Method) were performed for nonparametric data. $P < 0.05$ was considered statistically significant. Data are presented as mean ± SE.

[5] P. A. Masso-Welch, D. Zangani, C. Ip, M. M. Vaughan, S. Shoemaker, R. Chang, and M. M. Ip. Anti-angiogenic modulation of the mammary stromal-vascular environment by the cancer chemoprevention agent conjugated linoleic acid (CLA), manuscript in preparation.

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RESULTS

CLA Inhibits the Formation of Functional Blood Vessels *in Vivo*. s.c. injection of EHS-RBM supplemented with bFGF and heparan sulfate results in the formation of interconnecting networks of cells within the pellet, 1 week after injection (Fig. 1, *arrowheads*). A fibrocellular capsule containing multiple cell types (predominantly fibroblasts and polymorphonuclear cells), extracellular matrix, and blood vessels forms around the pellet (Fig. 1, *A, B, E,* and *F, arrows*). At higher magnification, it can be seen that the cellular network in mice fed the control diet is composed of interconnecting tubules with patent lumen containing RBCs (Fig. 1, *C* and *D, black arrowheads*). In contrast, the cellular network in mice fed a diet with 1 or 2% CLA is largely composed of flattened, solid cords of cells, most of which have collapsed lumen and no RBCs within (Fig. 1, *G* and *H, green arrowheads*).

Fig. 2 shows the results of quantitative analysis of these pellets. The pellets of CLA-fed mice showed a significantly decreased cellular invasion, quantified as the number of nuclei/field in the invaded regions, compared with the pellets of mice fed control diets (Fig. 2*A*). Despite the decrease in total cellularity, the average measured depth of cellular infiltration of the pellet was not different in the control and CLA-fed mice (data not shown).

The abundance of functional capillaries (cellular structures containing RBCs) was quantified to determine the effects of dietary CLA on functional angiogenesis (*i.e.,* the development of blood-conducting tubules). Fig. 2*B* demonstrates that both the 1% CLA and 2% CLA diets significantly decreased functional angiogenesis within the EHS pellet, compared with mice fed the control diet. There was no significant difference between 1% and 2% CLA.

Because of the involvement of mast cells and eosinophils in angiogenesis, the effect of CLA on mast cell and eosinophil number was analyzed. Mast cells, defined by toluidine blue staining (36, 37), and H&E-stained eosinophils, identified by their characteristic polymorphonuclear shape and eosinophilic cytoplasm, were quantified within the pellet as well as in the capsule region surrounding the pellet. No consistent difference in mast cell or eosinophil frequency was observed (data not shown).

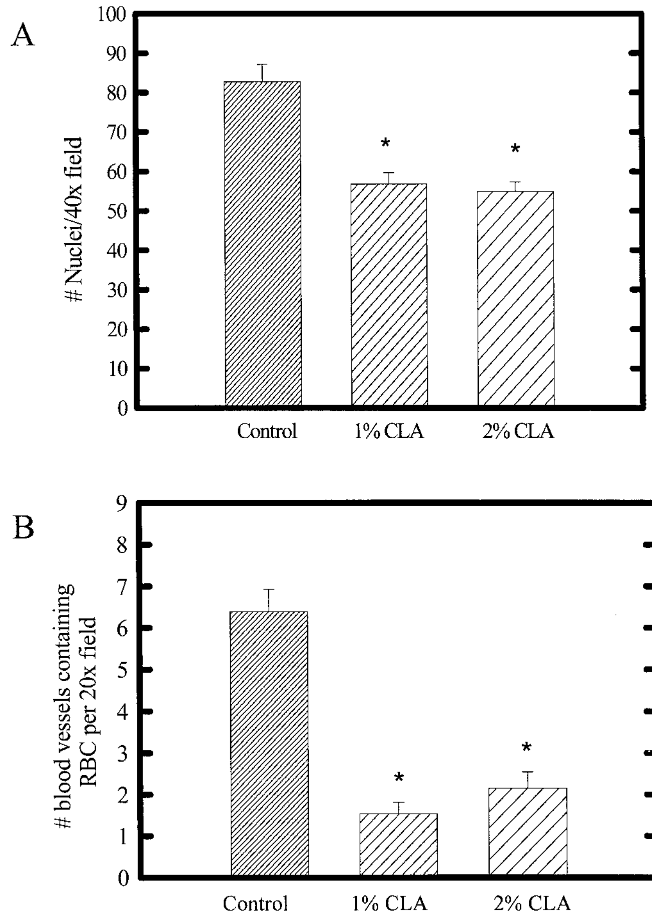

Fig. 2. Quantitation of the effects of dietary CLA on cellular invasion and functional angiogenesis. *A,* quantitation of cellularity within the pellet, performed by counting nuclei per 40× cell-invaded field. *B,* quantitation of blood vessels containing RBCs; both the 1% CLA and 2% CLA diets significantly decreased functional angiogenesis of the EHS pellets compared with mice fed the control diet. *, a statistically significant difference from control. *Bars,* SE.

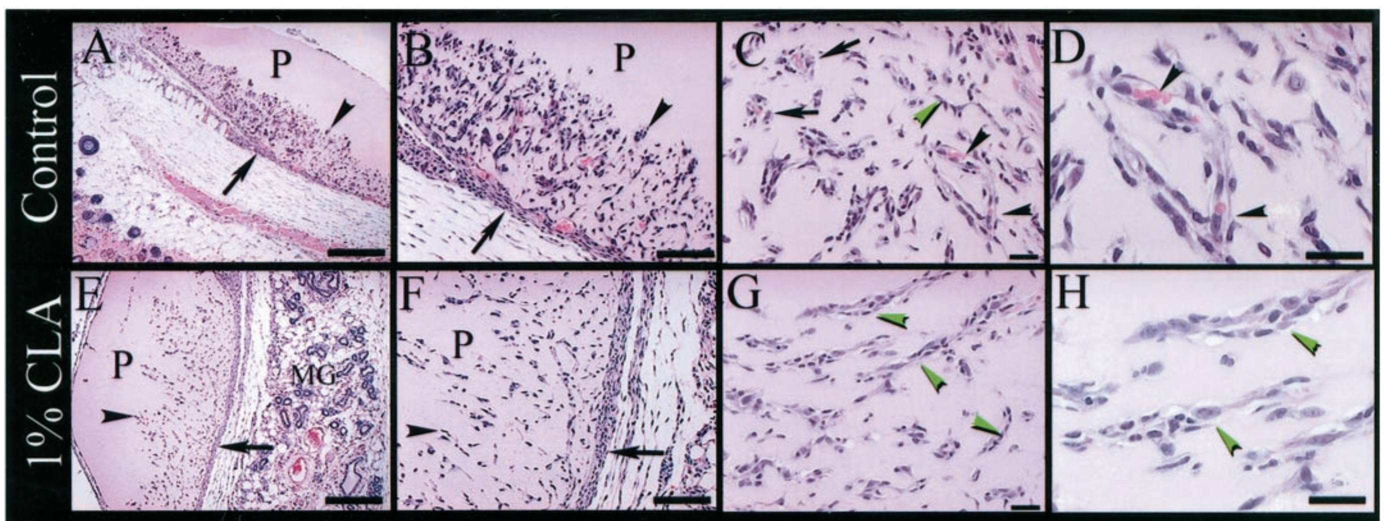

Fig. 1. Dietary CLA inhibits cellular recruitment and the formation of functional blood vessels in response to EHS-RBM angiogenic challenge. s.c. EHS-RBM injection of mice fed the control (*A–D*), 1% CLA (*E–H*), or 2% CLA (not shown) diets results in the formation of a fibrocellular capsule (*arrows*), from which cellular invasion (*arrowhead*) originates. *A* and *B* show the dense cellular infiltrate in mice fed the control diet. The cellular network in mice fed the 1% CLA diet (*E* and *F*) is considerably less dense. The cellular network in mice fed the control diet (*C* and *D*) is composed of interconnecting solid cords of cells (*green arrowheads*) or tubules with patent lumen containing RBCs (*black arrowheads,* longitudinal view; *black arrows* show a transverse view; *D* shows a higher magnification view of the same field in *C*). In contrast, the cellular network in mice fed a diet with 1% CLA is largely composed of solid cellular cords (*green arrowheads*), most of which have no lumen and no RBCs within (Fig. 1, *G* and *H*). *Bars: A* and *E,* 500 μm; *B* and *F,* 250 μm; *C, D, G,* and *H,* 50 μm. *P,* EHS-RBM pellet; *MG,* mammary gland.

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Dietary CLA Decreases VEGF Serum Levels and VEGF and Flk-1 Protein in the Mammary Gland. To determine how CLA might mediate its inhibitory effects on angiogenesis, serum VEGF-A (referred to hereafter as VEGF) concentrations were analyzed 7 days after angiogenic challenge (injection with EHS-RBM). Fig. 3 demonstrates that feeding with both the 1% CLA and 2% CLA diets for 7 weeks significantly reduced average serum VEGF levels by ~40%, relative to control diet.

This systemic effect of CLA on serum VEGF was only significant in mice stimulated by angiogenic challenge (pellet injection), which substantially increased baseline levels of VEGF (data not shown). If the effect of CLA were to be physiologically relevant, however, we would expect to see a local effect of dietary CLA on mammary gland VEGF, in the absence of angiogenic challenge. To examine this, mice were fed diets with or without CLA for 7 weeks, and whole mammary gland lysates were analyzed by Western blotting for VEGF. VEGF protein, detected as M_r 28,000 and M_r 54,000 reactive bands in these tissue lysates, was dramatically down-regulated in the mammary glands obtained from mice fed CLA (Fig. 4*A*). These results were quantified by scanning densitometry, and both bands were normalized to hsc70 levels to compensate for changes in cellularity of mammary glands from CLA-fed mice. As shown in Fig. 4*B*, both VEGF antibody-reactive bands were significantly decreased by dietary CLA in a dose-dependent manner.

Western blotting for Flk-1 was also performed to examine whether there was a coordinate down-regulation of VEGF receptor 2. As shown in Fig. 5*A*, Flk-1 protein was down-regulated by dietary CLA. Control diet-fed animals showed consistently high levels of both a M_r 200,000 Flk-1 antibody-reactive band, as well as a lower molecular weight (M_r 60,000) form. Both endothelial cells and 3T3 fibroblasts have been shown to express multiple processed forms of Flk-1 protein (38). Mice fed 1% CLA showed variable expression of Flk-1 protein, and animals fed 2% CLA showed uniformly low expression. Quantitation by scanning densitometry (Fig. 5*B*) demonstrated a statistically significant decrease in both the M_r 200,000 and M_r 60,000 Flk-1 antibody-reactive bands. This down-regulation occurred in a dose-dependent manner (Fig. 5*B*). Western blotting for Flt-1 was also conducted, but Flt-1 protein expression was not consistently detected in mammary gland lysates, using a variety of rat-reactive primary antibodies (39) and lysis conditions (data not shown).

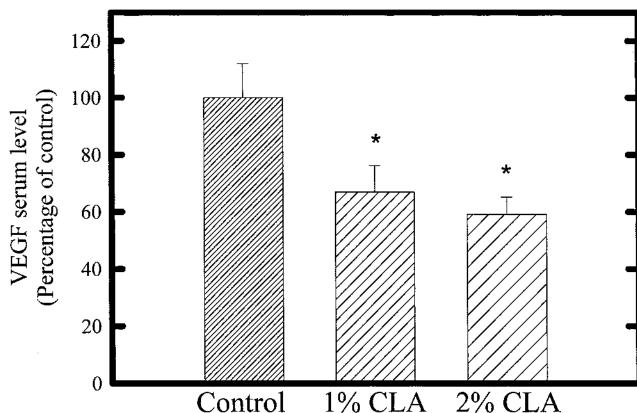

Fig. 3. Dietary CLA significantly decreases serum VEGF levels in mice that had received an EHS-RBM angiogenic challenge. Both the 1% and 2% CLA diets significantly decreased serum VEGF concentration in mice fed for 7 weeks, compared with control. Data (means; *bars,* SE) were obtained from four separate experiments (39 mice total for control diet, 39 mice total for 1% CLA, and 38 mice total for 2% CLA), each of which was normalized as percentage of control values for that experiment. *, significant differences from control ($P < 0.05$).

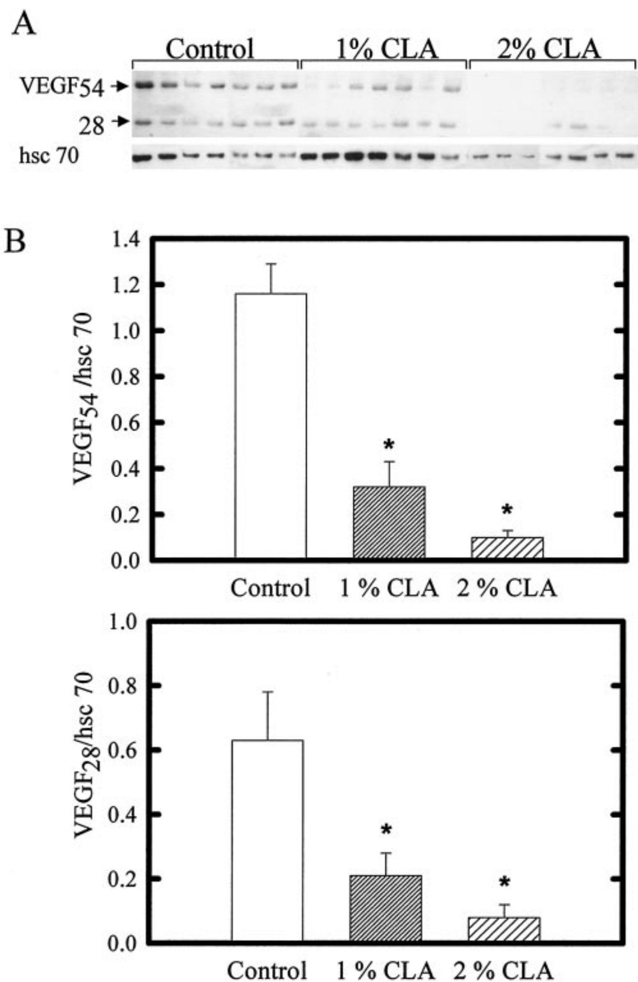

Fig. 4. Dietary CLA decreases VEGF protein in the mammary gland. Western blotting for VEGF in whole mammary gland lysates, loaded with equal protein/lane, is shown in *A*. Each lane represents a mammary gland lysate prepared from an individual mouse maintained on diet for 7 weeks, with no angiogenic challenge. Glands from seven mice/dietary group were analyzed. The blot was then stripped and reprobed for hsc70. *B,* quantification by scanning densitometry of the VEGF bands, normalized to hsc70, shows a dose-dependent, statistically significant decrease in both the higher (M_r 54,000) and lower (M_r 28,000) molecular weight forms of VEGF. *, values significantly different from control ($P < 0.05$). *Bars,* SE.

Purified CLA Isomers Inhibit the Formation of Microcapillary Networks *in Vitro*. To determine which isomer of CLA may be critical in mediating its effects *in vivo* and whether CLA is able to act directly on endothelial precursors, we used an *in vitro* angiogenesis assay to compare the ability of purified isomers of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, as well as the mixed isomer preparation, to affect microcapillary plexus formation by mammary stromal vascular cells (28). Both the total number of colonies (Fig. 6, *A–D*) and the size/extent of their branching into the angiogenic substrate (Fig. 6, *E–H*) were inhibited by the presence of CLA. Fig. 6*I* shows the quantification of the effect of CLA on microcapillary network size. Both mixed isomers and *trans*-10, *cis*-12 CLA significantly decreased network diameter at the three highest concentration tested (75, 100, and 125 μM). In contrast, *cis*-9, *trans*-11 CLA significant decreased network size at the two highest concentrations tested (100 and 125 μM).

DISCUSSION

Inhibition of Angiogenesis as One of the Mechanisms of the Antitumor Activity of CLA. CLA is truly a multipotent anticancer agent that can act at multiple levels of cancer development in multiple

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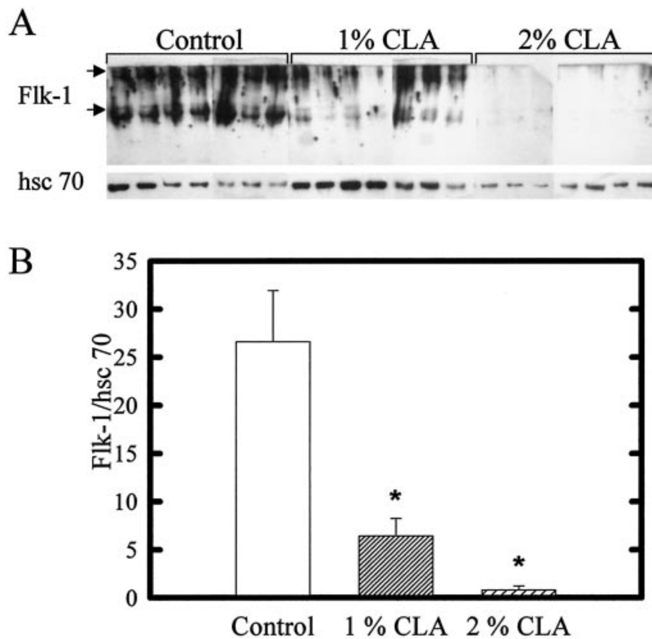
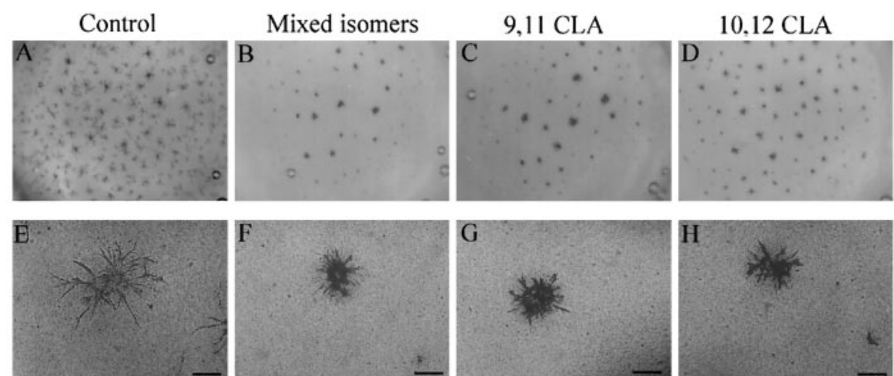


Fig. 5. Dietary CLA decreases Flk-1 protein in the mammary gland. Western blots of mammary gland lysates (Fig. 4) were reprobed by Western blotting for Flk-1. *A* shows that lysates from control-fed animals showed consistently high levels of Flk-1, with antibody-reactive bands at M_r 200,000 and M_r 60,000. In contrast, 1% CLA-fed mice showed variable expression. Animals fed 2% CLA showed uniformly low expression of Flk-1 protein. Glands from seven mice/dietary group were analyzed. Quantification by scanning densitometry (*B*) of both Flk-1 antibody-reactive bands, normalized to hsc70, demonstrated a dose-dependent, statistically significant decrease. ∗, values significantly different from control ($P < 0.05$). *Bars,* SE.

organ systems, including breast (9), skin (7), forestomach (5), and colon (6). CLA possesses multiple activities that could contribute to its antitumor efficacy, including antimutagenic activity (reviewed in Ref. 40), decreasing breast epithelial targets for transformation (14, 17), inhibition of initiation (2–6), promotion (7–9), progression (10), and metastasis (11–13). In addition to the ability of CLA to directly affect epithelial targets, the studies described here demonstrate that CLA can inhibit the process of angiogenesis *in vivo* and results in a decrease in systemic levels of VEGF and local levels of VEGF and its receptor Flk-1. This previously undescribed antiangiogenic effect may be a significant contributor to the antitumor effects of CLA. The previous observation that 1% CLA induces necrotic death in a prostate tumor model (11) is consistent with a vascular insufficiency induced by CLA. The antiangiogenic effects of dietary CLA described here peaked at 1%, similar to the peak effectiveness of 1% dietary CLA in its antitumor effects in mammary models (3).

Mechanisms of the Inhibitory Effect of CLA on Angiogenesis. On the basis of the results presented here, CLA inhibits the process of angiogenesis at several levels. CLA decreased the initial cellular recruitment or migration of stromal vascular precursors into the EHS-RBM, resulting in the overall decreased cellularity within the pellet (Figs. 1 and 2). Additionally, the cells that do enter the EHS-RBM plug in CLA-fed mice form solid cellular cords that resemble the immature vessels formed during embryonic vasculogenesis (reviewed in Ref. 41). CLA may therefore be interrupting or delaying the maturation of the cellular cords to mature vessels with patent lumen during morphogenesis of the newly formed tubules. Alternately, the absence of RBCs may reflect an inhibitory effect of CLA on the formation of connections of newly arising vessels in the EHS-RBM plug to the preexisting functional vasculature. The ability of blood



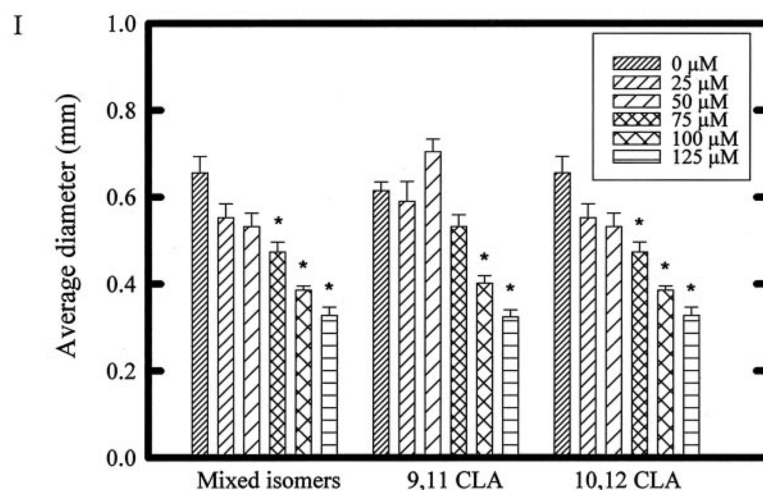


Fig. 6. Isomer dependence of CLA effects on endothelial differentiation *in vitro*. The effect of purified isomers of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA and mixed isomers to affect microcapillary network formation by stromal vascular precursors was compared. *A–D,* MTT staining of wells, showing individual networks formed by mammary stromal cells cultured on EHS. This dye is metabolized by viable cells; the total number of networks is decreased by all CLA isomers [100 μM of mixed (*B*), *cis*-9, *trans*-11 (*C*), and *trans*-10, *cis*-12 CLA (*D*)] tested, compared with control with no CLA (*A*). In addition to reducing the number of colonies, all CLA isomers (*F–H*) induced changes in mammary stromal cell capillary network morphology, with a darkening of the colony compared with control with no CLA and decreased branching compared with control (*E*). *I,* results of quantitation of the size (average diameter) of the branching microcapillary networks. ∗,statistically significant differences from control (no CLA). Each *column* represents the mean of 15 colonies from each treatment and concentration; *bars,* SE. *Bars:* E–H, 200 μM.

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flow to induce and in some cases precede "true" vessel formation has been described previously (42).

Some of these effects may be mediated by the down-regulation of systemic VEGF levels by dietary CLA (Fig. 3). VEGF is a heparin-binding cytokine that has been shown to stimulate vascular permeability and migration, proliferation, and apoptosis of endothelial cells (Ref. 43; reviewed in Ref. 44). A systemic effect of CLA on VEGF has not been described previously.

In addition to systemic effects, dietary CLA decreased local expression of VEGF protein within the mammary gland (Fig. 4). The importance of local VEGF in stimulating physiological angiogenesis in the rodent mammary gland is suggested by studies showing that VEGF is specifically up-regulated in whole mammary gland lysates during pregnancy (45). This increase in VEGF protein and mRNA has been localized to the lobular epithelium during pregnancy and lactation (46). CLA, via its direct effects on mammary epithelial cells, and indirectly through its effects on VEGF, has considerable potential as a multipotent chemopreventive agent. The down-regulation of VEGF within the mammary gland milieu is not only important because of its role as a growth factor for endothelial precursors. In addition to its effects on angiogenesis, VEGF has been shown stimulate the growth and invasiveness of breast cancer cells (47–51).

Cell types other than the mammary epithelium, such as macrophages, eosinophils, and mast cells, can synthesize and secrete VEGF (46, 51, 52) and may act as targets for the action of CLA as well. No reproducible effect of dietary CLA on mast cells or eosinophils associated with the EHS-RBM pellet or its surrounding capsule were seen (data not shown). Immunohistochemistry for VEGF, Flt-1, and Flk-1 was carried out to ascertain which cell types within the mammary gland were being affected by CLA. Despite a variety of pretreatments, primary antibodies, and detection methods tested, staining levels were too low to interpret or nonspecific compared with peptide inhibition controls (data not shown).

Modulation of the Mammary Gland Microenvironment by CLA. VEGF exerts its effects through a series of high-affinity receptors, including Flk-1 and Flt-1. Flk-1 (fetal liver kinase-1/VEGF receptor-2) is expressed early in endothelial differentiation (53), is required for embryonic vasculogenesis (54), and acts as the primary signaling receptor during angiogenesis in the adult. In the mammary gland, Flk-1 has been shown to be up-regulated during the rapid expansion of the lobular vascular bed that occurs during pregnancy/lactation (45, 46). This VEGF receptor has been localized via *in situ* hybridization to the mammary stromal cells of the fat pad as well as interstitial stroma (46). The dramatic loss of detectable Flk-1 in the mammary glands of mice fed a 2% CLA diet (Fig. 5) suggests that the stromal vascular precursors are a target of dietary CLA.

Direct, Isomer-specific Effects of CLA on Endothelial Precursors. A direct antiangiogenic effect of CLA on mammary stromal vascular precursor cells was demonstrated using the *in vitro* angiogenesis assay. Both the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers of CLA were able to act directly on the stromal vascular precursors *in vitro*, inducing the formation of fewer microcapillary networks with decreased branching and invasion of the surrounding EHS-RBM (Fig. 6).

Some of the effects of CLA on stromal cells differentiating *in vitro* to an endothelial phenotype may result from direct cellular cytotoxicity. Both the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isoforms of CLA, when added to mammary stromal vascular cells cultured on EHS, resulted in a greater than 50% decrease in MTT metabolism, even at concentrations as low as 25 μM CLA (data not shown), a concentration at which no significant effect on colony diameter is seen (Fig. 6). The fact that we begin to see antiangiogenic effects *in vitro* at concentrations of 75 μM suggests that endothelial cell-directed cytotoxicity is not the sole mechanism of the inhibitory effects of CLA on angiogenesis. It is noteworthy that the cytotoxic effect of low levels of CLA on mammary stromal cells *in vitro* is restricted to cells induced to differentiate into an endothelial phenotype by culture on EHS-RBM. The same concentrations of CLA used here (up to 125 μM) have no cytotoxic effects on the same mammary stromal vascular cells when cultured on plastic as fibroblast-like cells or when induced to differentiate to adipocytes.[6] Concentrations of CLA *in vitro* of up to 100 μM show no cytotoxicity in multiple nontransformed epithelial cell lines (55). Therefore, CLA may specifically decrease the survival of stromal cells that have recently undergone endothelial differentiation.

The *in vitro* inhibition of angiogenesis by CLA was relatively isomer independent. The *trans*-10, *cis*-12 CLA isomer was slightly more effective at 75 μM. The similar effects of these isomers in inhibiting angiogenesis may be physiologically relevant to chemoprevention, because both isomers are equally effective at inhibiting mammary carcinogenesis (56). In contrast, many other *in vivo* and *in vitro* metabolic effects of CLA show strict isomer dependence (reviewed in Ref. 1).

Efficacy of Dietary CLA as an Antiangiogenic Chemoprevention Agent at Physiological Doses. Inhibition of VEGF has been suggested as a therapeutic strategy for breast cancer (57). However, long-term chemopreventive modulation of the angiogenic reactivity and composition of normal breast tissue would require a more moderate approach. The ability of dietary CLA to decrease but not ablate angiogenesis *in vivo* suggests that it may be useful as a chemopreventive antiangiogenic agent. The ability of dietary CLA to act as a chemopreventive agent in human breast cancer has been suggested in several epidemiological studies, in which high dietary intake and tissue or serum CLA levels have been linked to a decreased breast cancer risk (58–60). Because none of the animal feeding studies has demonstrated toxicity, despite long-term feeding of CLA at high doses, CLA is generally regarded as safe (61), a prerequisite for long-term chemoprevention.

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[6] M. M. Ip, D. Zangani, D. Liu, L. Lee, P-P. Lee, and P. A. Masso-Welch. Conjugated linoleic acid induces a novel adipocyte differentiation pathway in mammary preadipocytes, manuscript in preparation.

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